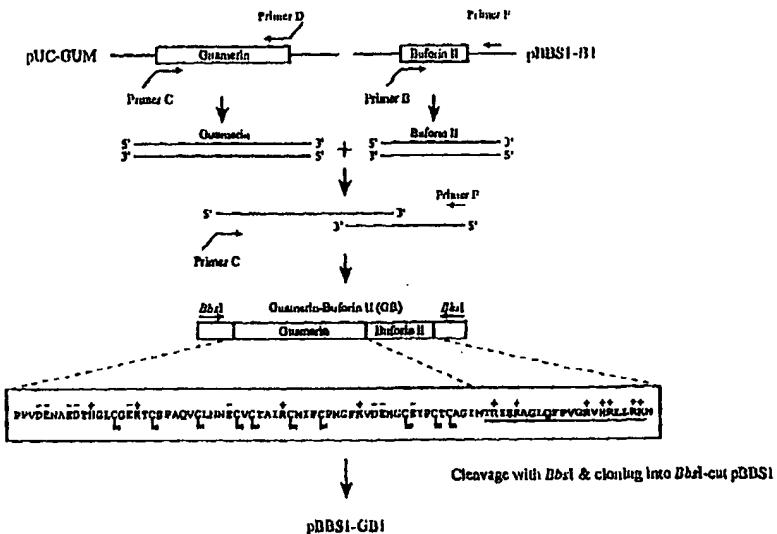




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(54) Title: METHOD FOR MASS PRODUCTION OF ANTIMICROBIAL PEPTIDE



(57) Abstract

The present invention provides a method for mass production of an antimicrobial peptide, which comprises the steps of: constructing a fusion gene containing a first gene encoding a negatively charged acidic peptide having at least two cysteine residues and a second gene encoding a positively charged basic antimicrobial peptide; transforming a host microorganism with an expression vector comprising the fusion gene; cultivating the transformed microorganism to express a fusion peptide containing the acidic peptide and the antimicrobial peptide; and, recovering the expressed antimicrobial peptide. In accordance with the present invention, the inhibitory effect of the expressed antimicrobial peptide on the growth of host microorganism can be dramatically minimized by fusing it with the acidic peptide. Accordingly, antimicrobial peptides can be produced massively from a recombinant microorganism regardless of the kind of the antimicrobial peptides.

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METHOD FOR MASS PRODUCTION OF ANTIMICROBIAL PEPTIDE

BACKGROUND OF THE INVENTION

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Field of the Invention

The present invention relates to a method for mass production of an antimicrobial peptide, more specifically, 10 to a method for mass production of an antimicrobial peptide by producing the antimicrobial peptide in a form of fusion protein with a foreign peptide through gene manipulation.

Description of the Prior Art

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In general, antimicrobial peptides do not easily lost their biological activities by physical and chemical factors such as heat, alkali, etc. Moreover, they do not readily induce a resistance to microorganisms as they show 20 an antimicrobial activity through their characteristic action mechanism which is clearly discriminated from conventional antibiotics. Thus, antimicrobial peptides have enjoyed high industrial applicability in the areas of pharmacy, food, etc.

25

However, there is a crucial problem in the industrial application of the antimicrobial peptides, since the conventional techniques do not permit mass production of the peptides in a low price. For example, chemical synthesis does not allow the mass production of the 30 antimicrobial peptides in an economical manner. In this regard, genetic engineering technology employing recombinant microorganisms, has been suggested in the art as an alternative means. However, it has also revealed a disadvantage of low productivity since the expressed 35 antimicrobial peptides inhibit the growth of the recombinant microorganisms.

USP 5,205,154 discloses a gene construct comprising a gene of a carrier polypeptide inhibiting the antimicrobial activity of cecropin and a gene of cecropin, where araB is employed as the carrier polypeptide, though the nature of the carrier polypeptide is not critical.

USP 5,593,866 teaches a process for preparing a positively charged antimicrobial peptide as a fusion protein with a negatively charged peptide to inhibit bacterial proteolysis, where glutathione-S-transferase, protein A, IgG-binding domain of protein A, protein F from Pseudomonas aeruginosa or prepro defensin is employed as the negatively charged peptide.

Accordingly, there are strong reasons for exploring and developing alternative means for mass production of the antimicrobial peptide in an economical manner.

SUMMARY OF THE INVENTION

The present inventors have made an effort to solve the disadvantages of low productivity and poor economy in the course of manufacturing the antimicrobial peptide, and successfully prepared the antimicrobial peptide in a massive and economical manner by the aid of recombinant DNA technology.

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A primary object of the present invention is to provide a method for mass production of an antimicrobial peptide in recombinant microorganisms, which employs an expression system permitting mass production of the antimicrobial peptide.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying

drawings, in which:

Figure 1(A) shows a nucleotide sequence (SEQ ID NO:1) of Guamerin gene and amino acid sequence translated therefrom (SEQ ID NO:2).

10 of MMIS(modified magainin intervening segment) gene and amino acid sequence translated therefrom(SEQ ID NO:4).

15 with a Buforin II gene by PCR.

20 product(SEQ ID NO:6) of a MMIS gene
with a Buforin II gene by PCR.

Figure 3 is a schematic diagram showing a construction strategy of a multimeric fusion gene using a gene amplification vector.

Figure 4(B) is a schematic diagram showing a construction strategy of a fusion gene(gene II) (SEQ ID NO:8) containing a gene of an antimicrobial peptide MSI-78 and a gene of Guamerin.

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Figure 5(A) is SDS-PAGE pattern of cell lysates of E. coli transformed with vectors containing multimeric fusion gene comprising Guamerin or MMIS after induction of protein expression.

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Figure 5(B) is SDS-PAGE pattern of cell lysates of E. coli transformed with vectors containing prepromagainin gene after induction of protein expression.

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Figure 6 is SDS-PAGE pattern of cell lysates of E. coli transformed with vectors comprising fusion genes containing Guamerin gene and genes of various antimicrobial peptides, after induction of protein expression.

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Figure 7 is SDS-PAGE pattern of cell lysates of E. coli transformed with vectors comprising fusion genes containing Guamerin gene and genes of various antimicrobial peptides, after induction of protein expression.

DETAILED DESCRIPTION OF THE INVENTION

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A method for mass production of an antimicrobial peptide of the present invention, comprises the steps of: constructing a fusion gene containing a first gene encoding an acidic peptide having at least two cysteine residues and 30 a second gene encoding a basic antimicrobial peptide; transforming a host microorganism with an expression vector comprising the fusion gene; culturing the transformed microorganism to express a fusion peptide containing the acid peptide and the antimicrobial peptide; and, recovering 35 the antimicrobial peptide from the fusion peptide.

In carrying out the present invention, a gene

construct which comprises a first gene encoding an acidic peptide having at least two cysteine residues and a second gene encoding a basic antimicrobial peptide, and an expression vector which comprises a promoter operably linked to a gene sequence containing a first gene encoding an acidic peptide having at least two cysteine residues and a second gene encoding a basic antimicrobial peptide, are essentially required, and the fusion gene may be present in a form of monomer or multimer.

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Contrary to the results in USP 5,593,866, the present inventors discovered that: a general acidic peptide gene does not permit an efficient expression of a basic antimicrobial peptide; and, the presence of at least two cysteine residues in the acidic peptide can efficiently solve the said problem.

In the gene construct of the invention, a first gene codes for an acidic peptide having at least two cysteine residues and neutralizing positive charges of an antimicrobial peptide substantially. Although the length of the acidic peptide is not limited, it is, preferably, equal to or longer than that of the antimicrobial peptide in order to efficiently neutralize the charges of a desired antimicrobial peptide, when considering length and distribution of positive charges. Furthermore, the acidic peptide has two or more cysteine residues. It is postulated that the cysteine residues promote interaction between negative charges of the acidic peptide and positive charges of the antimicrobial peptide by the formation of a secondary structure through disulfide bonds.

In accordance with the present invention, the acidic peptide may be synthesized artificially or selected among natural acidic peptides, and may be obtained using the synthetic gene encoding the peptide or isolated from nature. The artificially designed acidic peptide has two or more cysteine residues, and the natural acidic peptide may be

modified to have sufficient cysteine residues. Also, the acidic peptide gene may be modified in various ways for the purpose of easy fusion with a second gene encoding the antimicrobial peptide, easy isolation of the antimicrobial 5 peptide from the fusion peptide, or the preparation of various multimeric forms of a fusion gene.

For example, the acidic peptide gene may be synthesized or modified so that it can be connected to the antimicrobial peptide gene to have a correct reading frame 10 resulting the desired antimicrobial peptide. Also, the acidic peptide gene may be synthesized or modified to include nucleotide sequences encoding a cleavage site for a specific protease or a chemical in order to isolate the antimicrobial peptide from the expressed fusion peptide.

15 The acidic peptide gene may be selected to have the most suitable length for the neutralization of the antimicrobial peptide among monomers or multimers of the acidic peptide genes. The multimer of an acidic peptide gene may be prepared by employing gene amplification 20 technique. For example, vectors comprising multimers of an acidic peptide gene can be prepared by inserting an acidic peptide gene between two Class-IIS restriction enzyme sites of a vector containing two oppositely oriented Class-IIS restriction enzyme sites, digesting the vector with a 25 Class-IIS restriction enzyme, isolating a DNA fragment containing the acidic peptide gene, self-ligating the isolated DNA fragments to prepare multimers, and cloning the various multimers into the vector digested with the Class-IIS restriction enzyme(see: Lee, J.H. et al., *Genetic 30 Analysis: Biomolecular Engineering*, 13:139-145(1996)).

In accordance with the present invention, the antimicrobial peptide may be designed artificially or selected among natural acidic peptides, and may be obtained using the synthetic gene encoding the desired peptide or 35 isolated from nature. The antimicrobial peptide gene may be modified in various ways for the purpose of easy fusion

with the acidic peptide gene, easy isolation of the antimicrobial peptide from the fusion peptide, or the preparation of various multimeric forms of the fusion gene.

For example, the antimicrobial peptide gene may be 5 modified so that the C-terminal region of the antimicrobial peptide can be connected to the N-terminal region of the acidic peptide in a correct reading frame(antimicrobial peptide gene I).

Also, the antimicrobial peptide gene may be modified 10 to include nucleotide sequences encoding a site cleaved by a specific protease or a chemical at the N-terminus in order to isolate the antimicrobial peptide from the expressed fusion peptide, and nucleotide sequences permitting termination of peptide synthesis at the C-15 terminus of the antimicrobial peptide(antimicrobial peptide gene II).

In addition, the antimicrobial peptide gene may be modified to include nucleotide sequences encoding a cleavage site for a specific protease or a chemical(for 20 example, a codon encoding a methionine residue for the cleavage by CNBr) at the N-terminus and the C-terminus of the antimicrobial peptide in order to isolate the antimicrobial peptide from the expressed fusion peptide (antimicrobial peptide gene III).

25 The fusion gene may be prepared by ligating the acidic peptide gene and the antimicrobial peptide gene prepared as described above, and the acidic peptide gene or the antimicrobial peptide gene may be a monomer or a multimer as mentioned above.

30 In accordance with the present invention, the fusion gene contains a first gene encoding an acidic peptide and a second gene encoding an antimicrobial peptide gene, and those may be ligated directly or indirectly through linker, etc., if the two genes are connected in a correct reading 35 frame.

In a preferred embodiment of the invention, the fusion

gene may be prepared by modifying the acidic peptide gene and the antimicrobial peptide gene to have complementary nucleotide sequences at the 3'-termini of single strands of each gene, annealing two genes via a partial hybridization, 5 and performing PCR with the hybridized genes as a template and with primers corresponding to the sequences to the 5'-termini of respective single stranded genes.

Various number of monomer of a fusion gene thus prepared, may be concatenated to prepare various multimers 10 of the fusion gene by the conventional methods in the art, e.g., self-ligation of a fusion gene. A multimer of a fusion gene may be also prepared by employing gene amplification system. For example, vectors containing multimers of a fusion gene can be prepared by inserting the 15 fusion gene between two Class-IIS restriction enzyme sites of a vector containing two oppositely oriented Class-IIS restriction enzyme sites, digesting the vector with the Class-IIS restriction enzyme, isolating a DNA fragment containing the fusion gene, self-ligating the isolated DNA 20 fragments to prepare multimers of a fusion gene, and cloning the multimers of the fusion gene into the vector digested with the Class-IIS restriction enzyme.

In a preferred embodiment of the invention, a multimer 25 of a fusion gene is a multimer of the fusion gene comprising antimicrobial peptide gene III.

In a preferred embodiment of the invention, a multimer of a fusion gene is a multimer wherein the fusion gene comprising antimicrobial peptide gene II is ligated to 3'-terminus of a monomer or multimer of a fusion gene 30 comprising antimicrobial peptide gene I.

In a preferred embodiment of the invention, a multimer of a fusion gene is a multimer wherein the fusion gene comprising antimicrobial peptide gene II is ligated to 3'-terminus of a monomer or multimer of a fusion gene 35 comprising antimicrobial peptide gene III.

A multimer of a fusion gene can be cloned into a

suitable expression vector and expressed in a microorganism, — e.g., *E. coli*, to express multimer of the fusion peptide. The multimer of the fusion peptide is treated with an enzyme or a chemical, e.g., CNBr, to remove the acidic 5 peptide and separate the antimicrobial peptide into monomers, and the antimicrobial peptide is purified using cation-exchange chromatography, etc. When a multimer of a fusion peptide obtained after expression of a multimer is treated with an enzyme or a chemical, e.g., CNBr, the 10 antimicrobial peptide present at the end of the multimer may be obtained in a monomer of native form.

The present invention is further illustrated in the following examples, which should not be taken to limit the 15 scope of the invention. Particularly, since antimicrobial peptides, acidic peptides and genes of their multimers used in Examples are only preferred embodiments of the invention, the present invention covers all of the inventions employing an acidic peptide containing at least two 20 cysteins residues for the purpose of mass production of various basic antimicrobial peptides.

Example 1: Selection of acidic peptides

25 The native Guamerin(hereinafter, referred to as "G"; Jung, H.I. et al.(1995) *J. Biol. Chem.*, 270:13879-13884) and a modified MIS(hereinafter, referred to as "M"; Zasloff, M.(1987) *Proc. Natl. Acad. Sci., USA*, 84:5449-5453) which have a lot of cysteine residues were employed as acidic 30 peptides. As can be seen in Figures 1(A) and 1(B) (wherein only sense sequences were shown), single stranded oligonucleotides(SEQ ID NO:1; and, SEQ ID NO:3) encoding the acidic peptides were synthesized.

The oligonucleotides thus synthesized were dissolved 35 in TE buffer(pH 8.0) in the same molar ratio, heated at 70°C for 10 minutes, and left to stand at 0°C for 30

minutes. After 20% (w/v) polyacrylamide gel electrophoresis, double stranded DNA fragments were isolated, and cloned into pBBS1 vector (see: Lee, J.H. et al., *Genetic Analysis: Biomolecular Engineering*, 13:139-145 (1996)) digested with 5 BbsI to construct pBBS1-G₁ (Guamerin) or pBBS1-M₁ (MIS) vector. Since, multimers (pBBS1-G_n or pBBS1-M_n, n=1, 2, 3, ...) of the acidic peptide genes can be prepared using the pBBS1-G₁ or pBBS1-M₁ vector thus constructed, the acidic peptides having the most suitable length to neutralize an 10 antimicrobial peptide were selected.

Example 2: Preparation of an antimicrobial peptide gene

In order to prepare an antimicrobial peptide by 15 expressing the antimicrobial peptide in a multimeric form of a fusion peptide and treating it with CNBr, methionine codons were introduced to both ends of a gene of Buforin II (TRSSRAGLQFPVGRVHRLLRK (SEQ ID NO:9); Park, C.B. et al., (1996) *Biochem. Biophys. Res. Comm.*, 218, 408-413), an 20 antimicrobial peptide.

A DNA sequence encoding Buforin II (hereinafter, referred to as "B") was synthesized and cloned into pBBS1 vector digested with BbsI to construct pBBS1-B1 vector. The resulting pBBS1-B1 vector contains a complete Buforin 25 II gene and two methionine codons at both ends of the Buforin II gene.

Example 3: Preparation of a fusion gene containing an acidic peptide gene and an antimicrobial peptide gene 30

In order to prepare a fusion gene containing the acidic peptide gene and the antimicrobial peptide gene obtained in Examples 1 and 2, PCR was carried out as 35 followings (see: Figures 2(A) and 2(B)): Using a couple of primers corresponding to 5'-end and 3'-end of the acidic

peptide(i. e., Guamerin) gene(primer 1: 5'- AAAGAAGACGGCCCCCGGTCGACGAGAATGCG-3' (SEQ ID NO:10) and primer 2: 5'-GCTGCTACGGGTATGATCCCCGCGCAGGT-3' (SEQ ID NO:11)), respectively, the Guamerin gene was amplified by 5 the aid of PCR technique. On the other hand, using a couple of primers corresponding to 5'-end and 3'-end of the antimicrobial peptide(Buforin II)(primer 3: 5'-ACCTGCGCGGGATCATGACCCGTAGCAGC-3' (SEQ ID NO:12) and primer 4: 5'-TGCATGCCTGCAGGTCAA-3' (SEQ ID NO:13), respectively, 10 the Buforin II gene was amplified by PCR.

The PCR products thus amplified were mixed in a same molar ratio and amplified again by PCR using primer 1(SEQ ID NO:10) and primer 4(SEQ ID NO:13). The PCR product thus obtained was digested with BbsI. Then, the fragments of 15 the fusion gene containing the Guamerin gene and the Buforin II gene were isolated and cloned into pBBS1 vector digested with BbsI to construct pBBS1-(GB)1 vector(see: Figure 2(A)).

And then, in order to prepare multimers of the fusion 20 gene employing gene amplification system, the pBBS1-(GB)1 vector was digested with BbsI and the fragments containing the fusion gene were isolated. The isolated DNA fragments were self-ligated to prepare multimers, and the various 25 multimers were cloned into pBBS1 vector digested with BbsI to construct vectors comprising multimers of the fusion gene which were designated as pBBS1-(GB)_n(n=1,2,3,4,...) (see: Figure 3).

On the other hand, pBBS1-(MB)1 vector and vectors comprising multimers of the fusion gene, pBBS1-(MB)_n(n=1,2,3,4,...) were constructed in the same manner as 30 mentioned above, except for employing MIS(containing S-S bond) as acidic peptide instead of Guamerin(see: Figure 2(B)).

Example 4: Preparation of a fusion gene for the expression of a native antimicrobial peptide and its multimers

5 The antimicrobial peptide obtained from the multimers of fusion gene prepared in Example 3 has a homoserine residue at their C-terminus. In order to prepare a native antimicrobial peptide which does not contain the homoserine residue, a fusion gene whose sequence was slightly modified
10 from that of the fusion gene prepared in Example 3 and its multimers were prepared as followings: For this purpose, Guamerin was used as the acidic peptide and MSI-78 (GIGKFLKKAKKFGKAFVKILKK-NH₂: SEQ ID NO:14) was used as the antimicrobial peptide, respectively.

15 Two kinds of antimicrobial peptide genes (hereinafter, referred to as "BI" and "BII", respectively) suitable for the purpose were prepared, where antimicrobial peptide gene I was prepared so that the peptide encoded by this gene may have no methionine residue at the N-terminus, and the C-
20 terminus can be in-frame fused to the following acidic peptide gene in a correct reading frame, and antimicrobial peptide gene II was prepared so that the peptide encoded by this gene may have one methionine residue at N-terminus and peptide synthesis may be
25 terminated at C-terminus.

30 The antimicrobial peptide genes I and II thus prepared were ligated with an acidic peptide gene, respectively, in the same manner as in Example 3 to prepare fusion genes, and cloned into pBBS1 vector digested with BbsI to construct pBBS1-(GBI)₁ and pBBS1-(GB II)₁ vectors, respectively (see: Figures 4(A) and 4(B)). Multimers of the GB I fusion gene were prepared from the pBBS1-(GB I)₁ vector employing gene amplification system, and monomer of the GB II fusion gene was ligated to the ends of the
35 multimers to construct vectors which were designated as pBBS1-[(GB I)_n(GB II)] (n=0,1,2,3,4,...).

Example 5: Expression and preparation of antimicrobial peptide

In order to express the multimers of the fusion genes cloned in the vectors prepared in Examples 3 and 4 in *E. coli*, the multimers were cloned into an expression vector pET21c (Novagen, USA) digested with BamHI/HindIII, and transformed into *E. coli* BL21(DE3) to express multimers of the fusion peptides. After the induction of expression of the multimers, the cells were harvested from the cultured media. The lysates thus obtained were analyzed by SDS-PAGE (see: Figure 5(A)). In Figure 5(A), lane M shows molecular weight marker; lane 1 shows cell lysates of *E. coli* which does not contain the expression vector; and, lanes 2 to 14 show cell lysates of *E. coli* transformed with pET21c, pET21c-B₁, pET21c-B₂, pET21c-B₄, pET21c-B₆, pET21c-(GB)₁, pET21c-(GB)₂, pET21c-(GB)₄, pET21c-(GB)₆, pET21c-(MB)₁, pET21c-(MB)₂, pET21c-(MB)₄, and pET21c-(MB)₆, respectively. As shown in Figure 5(A), it was found that the expression of the multimers remarkably increased compared to that of the multimers of Buforin II alone. Among the recombinant *E. coli* containing an expression vector, one recombinant showing maximum expression was finally selected.

Inclusion bodies of the multimer of the fusion peptide whose expression was confirmed as above were suspended in a solution containing 1N HCl and 6M guanidinium chloride, and treated with 1M CNBr. Then, the peptides were collected by reverse-phase concentration using Sep-Pak, and the antimicrobial peptides with positive charges were purified by QAE-Sephadex (Sigma Chemical Co., USA) anion-exchange chromatography. The antimicrobial peptide thus isolated was further purified by reverse-phase HPLC to obtain a pure recombinant antimicrobial peptide. The analysis of biological activity of the purified recombinant antimicrobial peptide has revealed that it has the same

antimicrobial activity as that of the native one.

Comparative Example 1:

5 A gene encoding prepromagainin(SEQ ID NO:15) which has a similar structure to (MB)₆ but contains no cysteine residue, was cloned into a pET21b(Novagen, USA) vector and transformed into E. coli BL21(DE3). The nucleotide sequence and amino acid sequence translated therefrom are
10 as followings (wherein, the underlined sequence is magainin 1 or magainin 2):

Prepromagainin

15 ccaaaggcctctgcggatgaagatatggatgaaagagaggtccggggattgggt
P K A S A D E D M D E R E V R G I G
aaatttttgcattcagcggcaatttggaaaagctttgtggagagataatg
K F L H S A G K F G K A F V G E I M
aagtcaaaacgagatgcagaagcagtaggaccagaggccttgcagatgaagat
20 K S K R D A E A V G P E A F A D E D
tttagatgaaagagaggtccggggattggtaaattttgcactcagaaaaaaaa
L D E R E V R G I G K F L H S A K K
tttggaaaagctttgtggagagataatgaattcaaaacgagatgcagaagca
F G K A F V G E I M N S K R D A E A
25 gtaggaccagaggccttgcagatgaagattagatgaaagagaggtccgggg
V G P E A F A D E D L D E R E V R G
attggtaaattttgcactcagaaaaaaaaattggaaaagctttgtggagaa
I G K F L H S A K K F G K A F V G E
ataatgaattcaaaacgagatgcagaagcagtaggaccagaggccttgcagat
30 I M N S K R D A E A V G P E A F A D
gaagattagatgaaagagaggtccggggattggtaaattttgcactcagca
E D L D E R E V R G I G K F L H S A
aaaaaaaaattggaaaagctttgtggagaaataatgaattcaaaacgagatgca
K K F G K A F V G E I M N S K R D A
35 gaagcagtaggaccagaggccttgcagatgaagattagatgaaagagaggtc
E A V G P E A F A D E D L D E R E V

cggggaattggtaaattttgactcagaaaaattggaaaagctttgtg
R G I G K F L H S A K K F G K A F V
ggagagataatgaattcaaaacgagatgcagaagcagtaggaccagaggcctt
G E I M N S K R D A E A V G P E A F
5 gcagatgaagattttagatgaaagagaggtccgggaattggtaaattttgcac
A D E D L D E R E V R G I G K F L H
tcagcaaaaaattggaaaagctttgtggagagataatgaattcaaaacga
S A K K F G K A F V G E I M N S K R
gatgcagaagcagta (SEQ ID NO:15)
10 D A E A V (SEQ ID NO:16)

The cultured transformants were harvested and lysed. The lysates thus obtained were analyzed by the aid of SDS-PAGE (see: Figure 5(B)). In Figure 5(B), lane M shows 15 molecular weight markers (97.4, 66.2, 45, 31, 21.5, 14.4 Kd); lanes 1 and 2 show cell lysates of *E. coli* transformed with pET21b before and after IPTG induction; lanes 3 and 4 show cell lysate of *E. coli* transformed with pET21b-(prepromagainin) before and after IPTG induction. As shown 20 in Figure 5(B), it was found that expression of prepromagainin was not observed when the prepromagainin without cysteine residue was expressed using the same expression system.

25 Comparative Example 2:

A fusion gene was constructed with glutathione-S-transferase (GST) sequence and prepro defensin sequence from HNP-I as an acidic peptide gene, and PGQ, as an 30 antimicrobial peptide gene.

The preprodefensin sequence and the GST gene were treated with BsPLU11I and NcoI, respectively, and ligated with PGQ gene digested with NcoI. One methionine residue was incorporated between the acidec peptide and the 35 antimicrobial peptide for further cleavage with CNBr. The obtained fusion genes were cloned into pRSET (Invitrogen,

USA) vector and transformed into *E. coli* HMS174(DE3). The expression of the fusion peptides were analyzed by SDS-PAGE (see: Figure 6). In Figure 6, lane M shows molecular weight markers (97.4, 66.2, 45, 31, 21.5, 14.4 Kd); lane 1 shows cell lysates of *E. coli* HMS174(DM3); lanes 2 and 3 show cell lysates of *E. coli* harboring the vectors having the fusion genes of prepro definsin-PGQ and GST-PGQ.

Growth of *E. coli* cells harboring the vectors having the above fusion genes was severely inhibited. As shown in Figure 6, it was found that the expression of fusion peptide was not observed with prepro defensin as acidic peptide, while very low expression was observed with GST as acidic peptide.

15 Example 6: Preparation of a fusion gene comprising a Guamerin gene as an acidic peptide gene

The nucleotide sequence (SEQ ID NO:1) encoding Guamerin was slightly modified so that its C-terminus can be 20 digested with BspHI, and a methionine codon can be inserted in front of the antimicrobial peptide gene, when the guamerin gene is fused to the antimicrobial peptide gene in order to isolate only a pure antimicrobial peptide by CNBr cleavage of the fusion peptide.

25 First of all, an N-terminal oligonucleotide containing BamHI and NdeI restriction enzyme sites and a C-terminal oligonucleotide containing BamHI and BspHI restriction enzyme sites were synthesized as followings:

30 N-terminal oligonucleotide:

5'-CGGGATCCATATGCCCGGGTCGAC-3' (25mer)
(SEQ ID NO:17);

C-terminal oligonucleotide:

35 5'-CGGGATCCTCATGATAACCGCGCAG-3' (25mer)
(SEQ ID NO:18)

PCR was carried out by using the N- and C-terminal oligonucleotide primers thus synthesized and the Guamerin gene (Figure 1) as template, to synthesize a novel Guamerin gene.

5 Eight antimicrobial peptides known in the art (see: Peptide Science, Vol. 37, 105-122 (1995)) were selected to express them in a form of fusion peptide, whose biochemical characteristics are summarized in Table 1. The following DNA sequences (SEQ ID NO:19; SEQ ID NO:21; SEQ ID NO:23; SEQ
10 ID NO:25; SEQ ID NO:27; SEQ ID NO:29; SEQ ID NO:31; SEQ ID NO:33; SEQ ID NO:35) were deduced from the respective peptide sequences (SEQ ID NO:20; SEQ ID NO:22; SEQ ID NO:24; SEQ ID NO:26; SEQ ID NO:28; SEQ ID NO:30; SEQ ID NO:32; SEQ ID NO:34; SEQ ID NO:36) based on the codon usage of *E. coli*,
15 and synthesized for late use.

Apidaecin I

ggt aac aac cgt ccg gtt tac atc ccg cag ccg cgt ccg ccg
20 G N N R P V Y I P Q P R P P
cac ccg cgt act (SEQ ID NO:19)
H P R I (SEQ ID NO:20)

Bombinin

25 ggt atc ggt gcg ctg tct gcg aaa ggt gcg ctg aaa ggt ctg
G I G A L S A K G A L K G L
gcg aaa ggt ctg gcg gaa cac ttc gcg aac (SEQ ID NO:21)
A K G L A E H F A N (SEQ ID NO:22)

30

Cecropin A

aaa tgg aaa ttc aaa aaa atc gaa aaa gtt ggt cag aac atc
K W K F K K I E K V G Q N I
35 cgt gac ggt atc atc aaa gcg ggt ccg gcg gtt gcg gtt gtt
R D G I I K A G P A V A V V

ggt cag gcg acc cag atc gcg aaa (SEQ ID NO:23)
G Q A T Q I A K (SEQ ID NO:24)

Drosocin

5

ggt aaa ccg cgt ccg tac tct ccg cgt ccg acc tct cac ccg
G K P R P Y S P R P T S H P
cgt ccg atc gcg gtt (SEQ ID NO:25)
R P I A V (SEQ ID NO:26)

10

HNP-I

15 gcg tgc tac tgc cgt atc ccg gcg tgc atc gcg ggt gag cgt
A C Y C R I P A C I A G E R
cgt tac ggt acc tgc atc tac cag ggt cgt ctg tgg gcg ttc
R Y G T C I Y Q G R L W A F
tgc tgc (SEQ ID NO:27)
C C (SEQ ID NO:28)

20 Indolicidin

25 atc ctg ccg tgg aaa tgg ccg tgg tgg ccg tgg cgt cgt
I L P W K W P W W P W R R
(SEQ ID NO:29)
(SEQ ID NO:30)

Magainin (MSI-344)

30 ggt atc ggc aaa ttc ctg aaa aag gct aag aaa ttt ggt aag
G I G K F L K K A K K F G K
gcg ttc gtt aaa atc ctg aaa aag (SEQ ID NO:31)
A F V K I L K K (SEQ ID NO:32)

Melittin

ggt act ggt gcg gtt ctg aaa gtt ctg acc acc ggt ctg ccg
 G I G A V L K V L T T G L P
 5 gcg ctg atc tct tgg atc aaa cgt aaa cgt cag cag
 A L I S W I K R K R Q Q
 (SEQ ID NO:33)
 (SEQ ID NO:34)

10 Tachyplesin I

aaa tgg tgc ttc cgt gtt tgc tac cgt ggt atc tgc tac cgt
 K W C F F V C Y R G I C Y R
 cgt tgc cgt
 15 R C R (SEQ ID NO:35)
 (SEQ ID NO:36)

Table 1: Biochemical characteristics of various antimicrobial peptides*

20

| Peptides | Number of amino acid | Molecular weight(kDa) | pI | Origin |
|--------------------|----------------------|-----------------------|-------|--------|
| Apidaecin I | 18 | 2.1 | 12.21 | Insect |
| Bombinin | 24 | 29 | 10.34 | Frog |
| Ceropin A | 36 | 3.89 | 10.89 | Moth |
| Drosocin | 19 | 2.11 | 12.22 | Fly |
| HNPI | 30 | 3.46 | 8.28 | Human |
| Indolicidin | 13 | 1.91 | 12.51 | Cow |
| Magainin (MSI-344) | 22 | 2.48 | 11.41 | Frog |
| Melittin | 26 | 2.85 | 12.53 | Insect |
| Tachyplesin I | 17 | 2.27 | 10.01 | Crab |

*: Excerpted from Peptide Science, Vol 37, 105-122 (1995)

Various Guamerin-antimicrobial peptide fusion genes were prepared by fusing the synthesized Guamerin gene with 25 various antimicrobial peptide genes shown in Table 1,

respectively. That is, the synthesized Guamerin gene was digested with BspHI to give the termini complementary to BspHI or NcoI cleavage site, and fused with the antimicrobial peptide genes synthesized which were digested 5 with NcoI to prepare fusion genes.

Example 7: Expression of antimicrobial peptide

In order to express the fusion genes prepared in 10 Example 6 in *E. coli*, pRSET(Invitrogen, USA) expression vector was employed. The expression vector was digested with BamHI and EcoRI, dephosphorylated, and Guamerin-antimicrobial peptide fusion genes synthesized in Example 6 were cloned. *E. coli* BL21(DE3)pLysS was transformed with 15 the vectors having fusion genes by CaCl_2 method(see: Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (1989)).

The transformants were cultured in 5ml of LB medium supplemented with ampicillin at 37°C overnight. The 20 cultured cells were diluted in 5ml of fresh LB medium in a final concentration of 1%(v/v), and incubated at 37°C for 2 hours. Then, lactose was added to the cultured medium in a final concentration of 2% to induce the expression of the fusion peptides at 37°C for 4 hours. The expression of 25 fusion genes was analyzed by SDS-PAGE(see: Figure 7). In Figure 7, lane M shows molecular weight markers (97.4, 66.2, 45, 31, 21.5, 14.4 Kd); lanes 1-8 show cell lysates of *E. coli* transformed with fusion genes in which genes encoding apidaecin I, bombinin, cecropin A, drosocin, HNP1, 30 indolicidin, melittin and tachyplesin I were employed as antimictobial peptide genes, respectively.

As clearly illustrated and demonstrated as above, the present invention provides a method for mass production of 35 antimicrobial peptide, which comprises a step of preparing the antimicrobial peptide as a fusion peptide with a

foreign peptide. In accordance with the present invention, —
the inhibitory effect of the expressed antimicrobial
peptide on the growth of host microorganism can be
dramatically minimized by fusing it with the acidic peptide.
5 Accordingly, antimicrobial peptides can be produced
massively from a recombinant microorganism regardless of
the kind of the antimicrobial peptides.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

- (A) NAME: SAMYANG GENEX CORPORATION et al.
- (B) STREET: 263, Yonji-Dong, Chongno-Ku
- (C) CITY: Seoul
- (D) STATE: not applicable
- 10 (E) COUNTRY: Korea
- (F) POSTAL CODE (ZIP): 110-470
- (G) TELEPHONE: 042-865-8305
- (H) TELEFAX: 042-865-8399

15 (ii) TITLE OF INVENTION: METHOD FOR MASS PRODUCTION OF
ANTIMICROBIAL PEPTIDE

(iii) NUMBER OF SEQUENCES: 36

20 (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
25 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 183 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(vii) IMMEDIATE SOURCE:

40

- (B) CLONE: Guamerin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCCCGGTCTG ACGAGAATGC GGAGGACACA CATGGTCTCT GCGGGGAAAA 50
45 AACCTGCTCT CCAGCACAAAG TCTGTCTAAA CAACGAATGC GTTTCACTG 100
CAATCAGATG CGAGATCTTC TGTCTAACG GATTCAAAGT TGATGAGAAT 150
50 GGATGCGAAT ACCCATGTAC CTGCGCGGGG ATC 183

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 61 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear
(D) MOLECULE TYPE: peptide

10 (vii) IMMEDIATE SOURCE:
(B) CLONE: Guamerin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

15 Pro Pro Val Asp Glu Asn Ala Glu Asp Thr His Gly Leu Cys
1 5 10

20 Gly Glu Lys Thr Cys Ser Pro Ala Gln Val Cys Leu Asn Asn
15 20 25

25 Glu Cys Val Cys Thr Ala Ile Arg Cys Glu Ile Phe Cys Pro
30 35 40

Asn Gly Phe Lys Val Asp Glu Asn Gly Cys Glu Tyr Pro Cys
45 50 55

25 Thr Cys Ala Gly Ile
60

(2) INFORMATION FOR SEQ ID NO:3:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
35 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

40 (vii) IMMEDIATE SOURCE:
(B) CLONE: MIS(magainin intervening segment) gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

45 CCCCTGTGCG ATGCAGAAGC AGTAGGACCA GAGGCCTTG CAGATGAAGA 50

TTTAGATGAA TGCCCCGGG TCTTCTAGAG T 81

50 (2) INFORMATION FOR SEQ ID NO:4:

5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

10 (vii) IMMEDIATE SOURCE:
(B) CLONE: MIS

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15 Pro Leu Cys Asp Ala Glu Ala Val Gly Pro Glu Ala Phe Ala
1 5 10

15 Asp Glu Asp Leu Phe Ala Asp Glu Asp Leu Asp Glu Cys
15 20 25

20 (2) INFORMATION FOR SEQ ID NO:5:

25 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 84 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: protein

30 (vii) IMMEDIATE SOURCE:
(B) CLONE: Guamerin/BuforinII fusion protein

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

35 Pro Pro Val Asp Glu Asn Ala Lys Asp Thr His Gly Leu Cys
1 5 10

35 Gly Glu Lys Thr Cys Ser Pro Ala Gln Val Cys Leu Asn Asn
15 20 25

40 Glu Cys Val Cys Thr Ala Ile Arg Cys Met Ile Phe Cys Pro
30 35 40

45 Asn Gly Phe Lys Val Asp Lys Asn Gly Cys Glu Tyr Pro Cys
45 50 55

45 Thr Cys Ala Gly Ile Met Thr Arg Ser Ser Arg Ala Gly Leu
60 65 70

50 Gln Phe Pro Val Gly Arg Val His Arg Leu Leu Arg Lys Met
75 80

50 (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

10 (B) CLONE: MIS/BuforinII fusion protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

15 Pro Leu Cys Asp Ala Lys Ala Val Gly Pro Glu Ala Phe Ala
1 5 10

Asp Glu Asp Leu Asp Glu Cys Pro Leu Met Thr Arg Ser Ser
15 20 25

20 Arg Ala Gly Leu Gln Phe Pro Val Gly Arg Val His Arg Leu
30 35 40

Leu Arg Lys Met
45

25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

35

(vii) IMMEDIATE SOURCE:

(B) CLONE: Guamerin/MSI-78 fusion protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

40 Pro Pro Val Asp Glu Asn Ala Glu Asp Thr His Gly Leu Cys
1 5 10

Gly Glu Lys Thr Cys Ser Pro Ala Gln Val Cys Leu Asn Asn
15 20 25

45

Glu Cys Val Cys Thr Ala Ile Arg Cys Glu Ile Phe Cys Pro
30 35 40

Asn Gly Phe Lys Val Asp Glu Asn Gly Cys Glu Tyr Pro Cys
50 55

Thr Cys Ala Gly Ile Cys Gly Ile Gly Lys Phe Leu Lys Lys
60 65 70

5 Ala Lys Lys Phe Gly Lys Ala Phe Val Lys Ile Leu Lys Lys
75 80

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 84 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

(B) CLONE: Guamerin/MSI-78 fusion protein

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

20 Pro Pro Val Asp Glu Asn Ala Glu Asp Thr His Gly Leu Cys
1 5 10

25 Gly Glu Lys Thr Cys Ser Pro Ala Gln Val Cys Leu Asn Asn
15 20 25

Glu Cys Val Cys Thr Ala Ile Arg Cys Glu Ile Phe Cys Pro
30 35 40

30 Asn Gly Phe Lys Val Asp Glu Asn Gly Cys Glu Tyr Pro Cys
45 50 55

Thr Cys Ala Gly Ile Met Gly Ile Gly Lys Phe Leu Lys Lys
60 65 70

35 Ala Lys Lys Phe Gly Lys Ala Phe Val Lys Ile Leu Lys Lys
75 80

(2) INFORMATION FOR SEQ ID NO:9:

40 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

(B) CLONE: BuforinII

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Arg Ser Ser Arg Ala Gly Leu Gln Phe Pro Val Gly Arg
1 5 10

5 Val His Arg Leu Leu Arg Lys
15 20

(2) INFORMATION FOR SEQ ID NO:10:

10 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAAGAAGACG GCCCCCGGTC GACGAGAATG CG 32

25 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

35 (vii) IMMEDIATE SOURCE:
(B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

40 GCTGCTACGG GTCATGATCC CCGCGCAGGT 30

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

45 (ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

5 ACCTGCGCGG GGATCATGAC CCGTAGCAGC 30

(2) INFORMATION FOR SEQ ID NO:13:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: primer

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGCATGCCTG CAGGTCGA 18

25 (2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

35 (B) CLONE: MSI-78

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

40 Gly Ile Gly Lys Phe Leu Lys Lys Ala Lys Lys Phe Gly Lys
1 5 10
Ala Phe Val Lys Ile Leu Lys Lys
15 20

(2) INFORMATION FOR SEQ ID NO:15:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 825 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
50 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

5 (vii) IMMEDIATE SOURCE:

(B) CLONE: prepromagainin gene

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

10 CCAAAGGCCT CTGCGGATGA AGATATGGAT GAAAGAGAGG TCCGGGAAAT 50
TGGTAAATTT TTGCATTCAG CGGGCAAATT TGAAAAGCT TTTGTGGGAG 100
AGATAATGAA GTCAAAACGA GATGCAGAAG CAGTAGGACC AGAGGCCTTT 150
15 GCAGATGAAG ATTTAGATGA AAGAGAGGTC CGGGGAATTG GTAAATTTTT 200
GCACTCAGCA AAAAAATTTG GAAAAGCTTT TGTGGGAGAG ATAATGAATT 250
20 CAAAACGAGA TGCAGAAGCA GTAGGACCAG AGGCCTTGCA AGATGAAGAT 300
TTAGATGAAA GAGAGGTCCG GGGATTGGT AAATTTTGCA ACTCAGCAAA 350
AAAATTTGGA AAAGCTTTG TGGGAGAAAT AATGAATTCA AAACGAGATG 400
25 CAGAACAGT AGGACCAGAG GCCTTGCAAG ATGAAGATT AGATGAAAGA 450
GAGGTCCGGG GAATTGGTAA ATTTTGCAAC TCAGCAAAAA AATTGGAAA 500
30 AGCTTTGTG GGAGAAATAA TGAATTCAAA ACGAGATGCA GAAGCAGTAG 550
GACCAGAGGC CTTTGCAGAT GAAGATTAG ATGAAAGAGA GGTCCGGGAA 600
ATTGGTAAAT TTTTGCACTC AGCAAAAAAA TTTGGAAAAG CTTTGTGGG 650
35 AGAGATAATG AATTCAAAAC GAGATGCAGA AGCAGTAGGA CCAGAGGCCT 700
TTGCAGATGA AGATTTAGAT GAAAGAGAGG TCCGGGAAAT TGGTAAATTT 750
40 TTGCACTCAG CAAAAAAATT TGGAAAAGCT TTTGTGGGAG AGATAATGAA 800
TTCAAAACGA GATGCAGAAG CAGTA 825

(2) INFORMATION FOR SEQ ID NO:16:

45 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 275 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: protein

(vii) IMMEDIATE SOURCE:

(B) CLONE: prepromagainin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

5 Pro Lys Ala Ser Ala Asp Glu Asp Met Asp Glu Arg Glu Val
1 5 10

Arg Gly Ile Gly Lys Phe Leu His Ser Ala Gly Lys Phe Gly
15 20 25

10 Lys Ala Phe Val Gly Glu Ile Met Lys Ser Lys Arg Asp Ala
30 35 40

Glu Ala Val Gly Pro Glu Ala Phe Ala Asp Glu Asp Leu Asp
15 45 50 55

Glu Arg Glu Val Arg Gly Ile Gly Lys Phe Leu His Ser Ala
60 65 70

20 Lys Lys Phe Gly Lys Ala Phe Val Gly Glu Ile Met Asn Ser
75 80

Lys Arg Asp Ala Glu Ala Val Gly Pro Glu Ala Phe Ala Asp
85 90 95

25 Glu Asp Leu Asp Glu Arg Glu Val Arg Gly Ile Gly Lys Phe
100 105 110

Leu His Ser Ala Lys Lys Phe Gly Lys Ala Phe Val Gly Glu
30 115 120 125

Ile Met Asn Ser Lys Arg Asp Ala Glu Ala Val Gly Pro Glu
130 135 140

35 Ala Phe Ala Asp Glu Asp Leu Asp Glu Arg Glu Val Arg Gly
145 150

Ile Gly Lys Phe Leu His Ser Ala Lys Lys Phe Gly Lys Ala
155 160 165

40 Phe Val Gly Glu Ile Met Asn Ser Lys Arg Asp Ala Glu Ala
170 175 180

Val Gly Pro Glu Ala Phe Ala Asp Glu Asp Leu Asp Glu Arg
45 185 190 195

Glu Val Arg Gly Ile Gly Lys Phe Leu His Ser Ala Lys Lys
200 205 210

50 Phe Gly Lys Ala Phe Val Gly Glu Ile Met Asn Ser Lys Arg
215 220

Asp Ala Glu Ala Val Gly Pro Glu Ala Phe Ala Asp Glu Asp
225 230 235

5

Leu Asp Glu Arg Glu Val Arg Gly Ile Gly Lys Phe Leu His
240 245 250

10

Ser Ala Lys Lys Phe Gly Lys Ala Phe Val Gly Glu Ile Met
255 260 265

Asn Ser Lys Arg Asp Ala Glu Ala Val
270 275

15 (2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- 20 (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

25 (vii) IMMEDIATE SOURCE:

- (B) CLONE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

30 CGGGATCCAT ATGCCCGGG TCGAC 25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- 35 (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA

(vii) IMMEDIATE SOURCE:

- (B) CLONE: primer

45 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGGGATCCTC ATGATAACCG CGCAG 25

(2) INFORMATION FOR SEQ ID NO:19:

50 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTAACAAACC GTCCGGTTTA CATCCCGCAG CCGCGTCCGC CGCACCCGCG 50

TACT

54

15

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
20 (B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

25

(vii) IMMEDIATE SOURCE:

(B) CLONE: Apidaecin I

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

30 Gly Asn Asn Arg Pro Val Tyr Ile Pro Gln Pro Arg Pro Pro
1 5 10

His Pro Arg Ile

15

35

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 72 base pairs
40 (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

45

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

50 GGTATCGGTG CGCTGTCTGC GAAAGGTGCG CTGAAAGGTC TGGCGAAAGG 50

TCTGGCGGAA CACTTCGCGA AC

72

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

10 (vii) IMMEDIATE SOURCE:
(B) CLONE: Bombinin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

15 Gly Ile Gly Ala Leu Ser Ala Lys Gly Ala Leu Lys Gly Leu
1 5 10

Ala Lys Gly Leu Ala Glu His Phe Ala Asn
20 15 20

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

35 AAATGGAAAT TCAAAAAAAT CGAAAAAGTT GGTCAAGAACCA TCCGTGACGG 50
TATCATCAAA GCGGGTCCGG CGGTTGCGGT TGTTGGTCAG GCGACCCAGA 100
40 TCGCGAAA 108

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

45 (A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (vii) IMMEDIATE SOURCE:

(B) CLONE: Cecropin A

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

5 Lys Trp Lys Phe Lys Lys Ile Glu Lys Val Gly Gln Asn Ile
1 5 10

Arg Asp Gly Ile Ile Lys Ala Gly Pro Ala Val Ala Val Val
15 20 25

10 Gly Gln Ala Thr Gln Ile Ala Lys
30 35

(2) INFORMATION FOR SEQ ID NO:25:

15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GGTAAACCGC GTCCGTACTC TCCGCGTCCG ACCTCTCACC CGCGTCCGAT 50
30 CGCGGTT 57

(2) INFORMATION FOR SEQ ID NO:26:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

40 (vii) IMMEDIATE SOURCE:

(B) CLONE: Drosocin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

45 Gly Lys Pro Arg Pro Tyr Ser Pro Arg Pro Thr Ser His Pro
1 5 10

Arg Pro Ile Ala Val
50 15

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

10

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

15 GCGTGCTACT GCCGTATCCC GGCGTGCATC GCGGGTGAGC GTCGTTACGG 50

TACCTGCATC TACCAGGGTC GTCTGTGGGC GTTCTGCTGC

90

(2) INFORMATION FOR SEQ ID NO:28:

20

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: linear

25

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

- (B) CLONE: HNP-I

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ala Cys Tyr Cys Arg Ile Pro Ala Cys Ile Ala Gly Glu Arg
1 5 10

35

Arg Tyr Gly Thr Cys Ile Tyr Gln Gly Arg Leu Trp Ala Phe
15 20 25Cys Cys
30

40

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

50

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATCCTGCCGT GGAAATGGCC GTGGTGGCCG TGGCGTCGT

39

5

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- 10 (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

15 (vii) IMMEDIATE SOURCE:

- (B) CLONE: Indolicidin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

20 Ile Leu Pro Trp Lys Trp Pro Trp Trp Pro Trp Arg Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:31:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 66 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGTATCGGCA AATTCTGAA AAAGGCTAAG AAATTTGGTA AGGCCTTCGT 50

TAAAAATCCTG AAAAAG

66

40

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- 45 (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

50 (vii) IMMEDIATE SOURCE:

- (B) CLONE: Magainin

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

5 Gly Ile Gly Lys Phe Leu Lys Lys Ala Lys Lys Phe Gly Lys
1 5 10
Ala Phe Val Lys Ile Leu Lys Lys
15 20

10 (2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 78 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (iv) ANTI-SENSE: NO

20 (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

25 GGTACTGGTG CGGTTCTGAA AGTTCTGACC ACCGGTCTGC CGGCGCTGAT 50
CTCTTGGATC AACCGTAAAC GTCAGCAG 78

(2) INFORMATION FOR SEQ ID NO:34:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: Melittin

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Gly Ile Gly Ala Val Leu Lys Val Leu Thr Thr Gly Leu Pro
1 5 10

45 Ala Leu Ile Ser Trp Ile Lys Arg Lys Arg Gln Gln
15 20 25

(2) INFORMATION FOR SEQ ID NO:35:

50 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

10 AAATGGTGCT TCCGTGTTG CTACCGTGTT ATCTGCTACC GTCGTTGCCG 50

T 51

15 (2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

25 (B) CLONE: Tachyplesin I

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

30 Lys Trp Cys Phe Phe Val Cys Tyr Arg Gly Ile Cys Tyr Arg
1 5 10

Arg Cys Arg
15

35

WHAT IS CLAIMED IS:

1. A method for mass production of antimicrobial peptide, which comprises the steps of:

5

- (i) constructing a fusion gene containing a first gene encoding a negatively charged acidic peptide having at least two cysteine residues and a second gene encoding a positively charged basic antimicrobial peptide;
- 10 (ii) transforming a host microorganism with an expression vector comprising the fusion gene;
- (iii) cultivating the transformed microorganism to express a fusion peptide containing the acidic peptide and the antimicrobial peptide; and,
- 15 (iv) recovering the expressed antimicrobial peptide.

2. The method of claim 1, wherein the fusion gene contains at least one cleavage site for a protease or a 20 chemical between the acidic peptide and the antimicrobial peptide.

3. The method of claim 1, wherein the negative charges of the acidic peptide substantially neutralizes the 25 positive charges of the antimicrobial peptide.

4. The method of claim 1, wherein the expression vector comprises a multimer of the fusion gene.

30 5. The method of claim 1, wherein the expression vector comprises a promoter operably linked to a gene encoding a fusion peptide which contains an acidic peptide neutralizing positive charges of an antimicrobial peptide and having at least two cysteine residues, and an 35 antimicrobial peptide.

6. The method of claim 5, wherein the gene is a —
multimeric form of a first gene encoding an acidic peptide
neutralizing positive charges of an antimicrobial peptide
and having at least two cysteine residues, and a second
5 gene encoding a positively charged antimicrobial peptide
which is fused to the first gene.

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5' CCC CCG GTC GAC GAG AAT GCG GAG GAC ACA
Pro Pro Val Asp Glu Asn Ala Glu Asp Thr

CAT GGT CTC TGC GGG GAA AAA ACC TGC TCT
His Gly Leu Cys Gly Glu Lys Thr Cys Ser

CCA GCA CAA GTC TGT CTA AAC AAC GAA TGC
Pro Ala Gln Val Cys Leu Asn Asn Glu Cys

GTT TGC ACT GCA ATC AGA TGC GAG ATC TTC
Val Cys Thr Ala Ile Arg Cys Glu Ile Phe

TGT CCT AAC GGA TTC AAA GTT GAT GAG AAT
Cys Pro Asn Gly Phe Lys Val Asp Glu Asn

GGA TGC GAA TAC CCA TGT ACC TGC GCG GGG ATC 3'
Gly Cys Glu Tyr Pro Cys Thr Cys Ala Gly Ile

FIG. 1(A)

5' CCC CTG TGC GAT GCA GAA GCA GTA GGA CCA
Pro Leu Cys Asp Ala Glu Ala Val Gly Pro

GAG GCC TTT GCA GAT GAA GAT TTA GAT GAA TGC 3'
Glu Ala Phe Ala Asp Glu Asp Leu Asp Glu Cys

FIG. 1(B)

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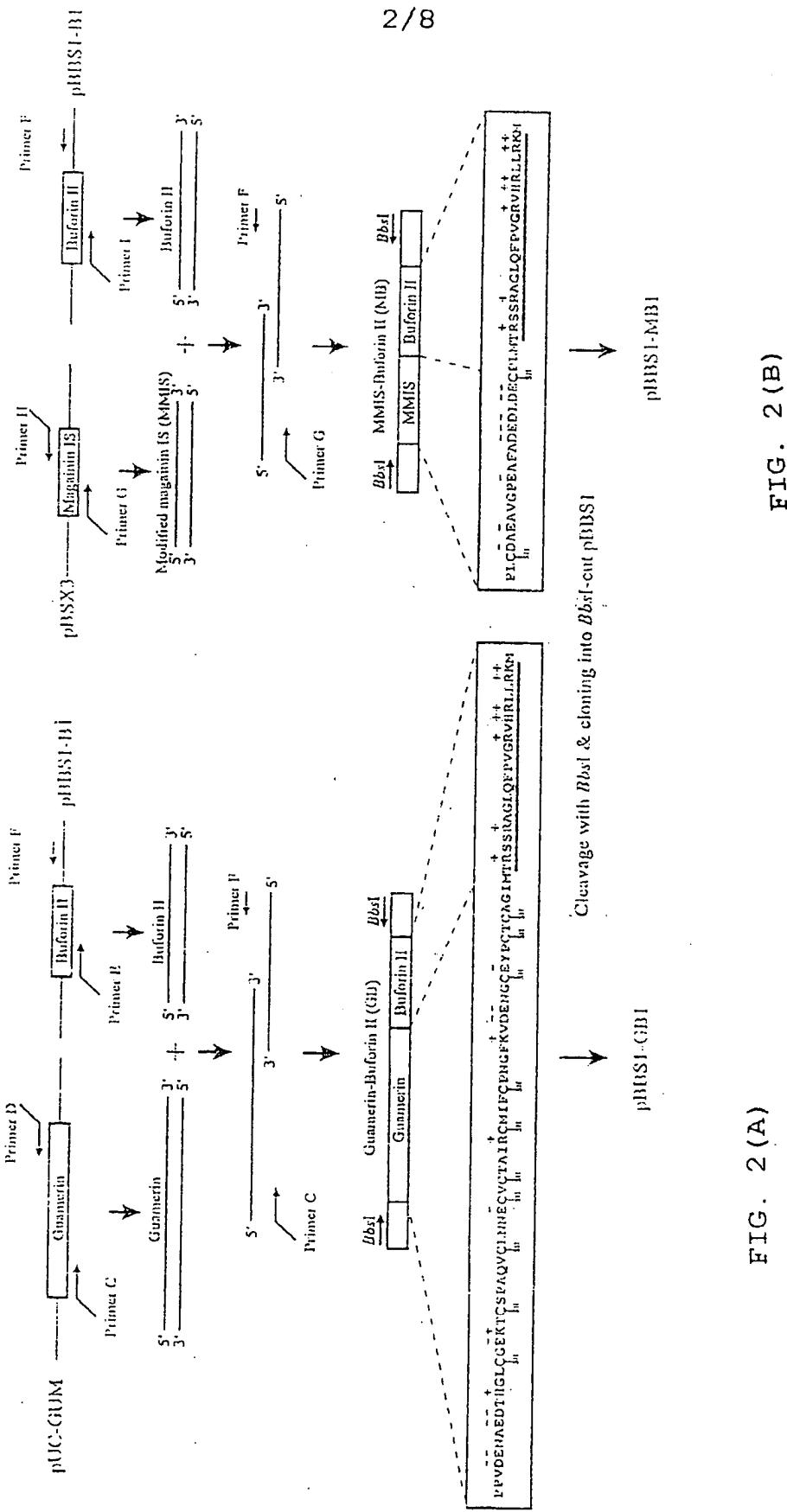


FIG. 2 (B)

FIG. 2 (A)

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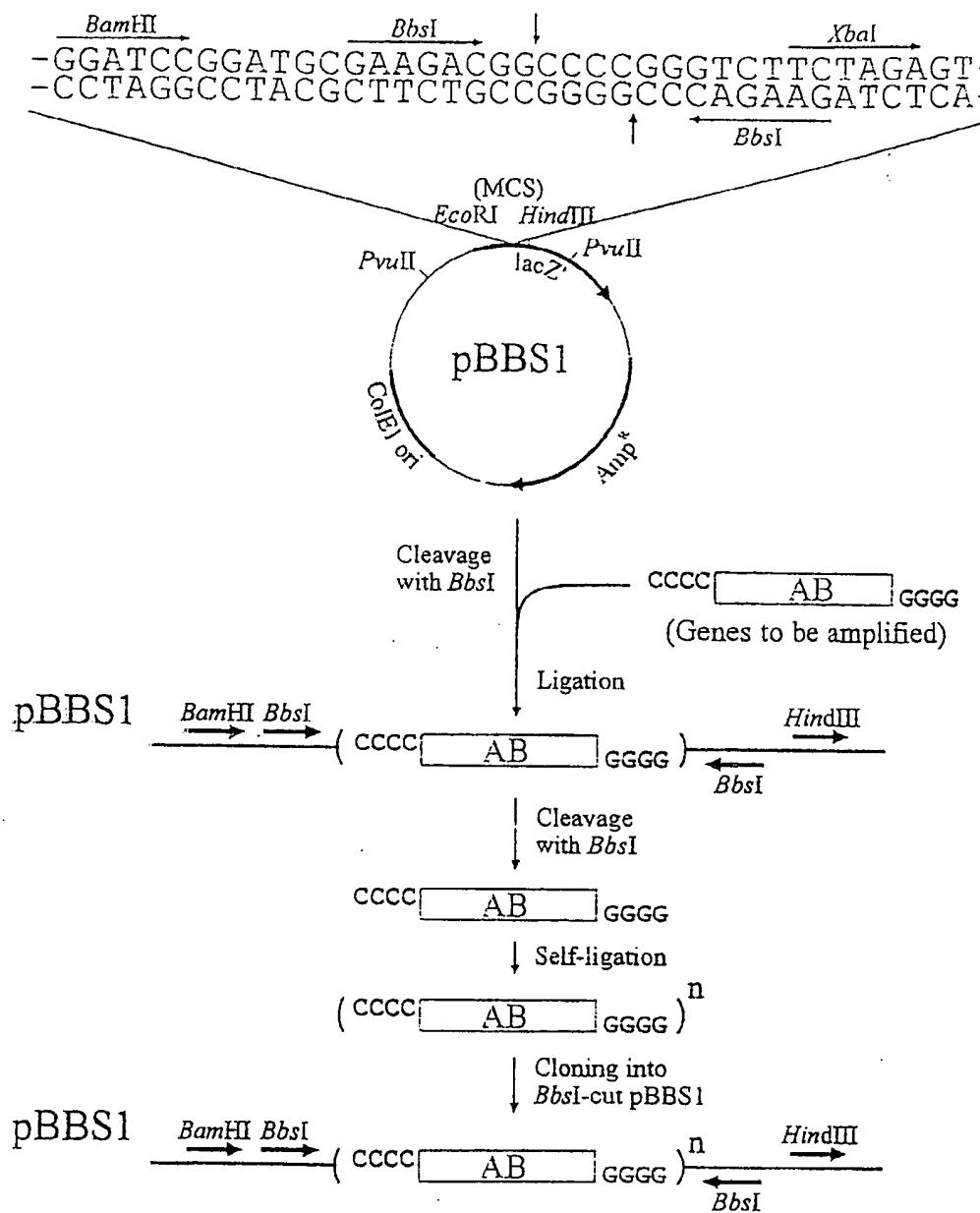


FIG. 3

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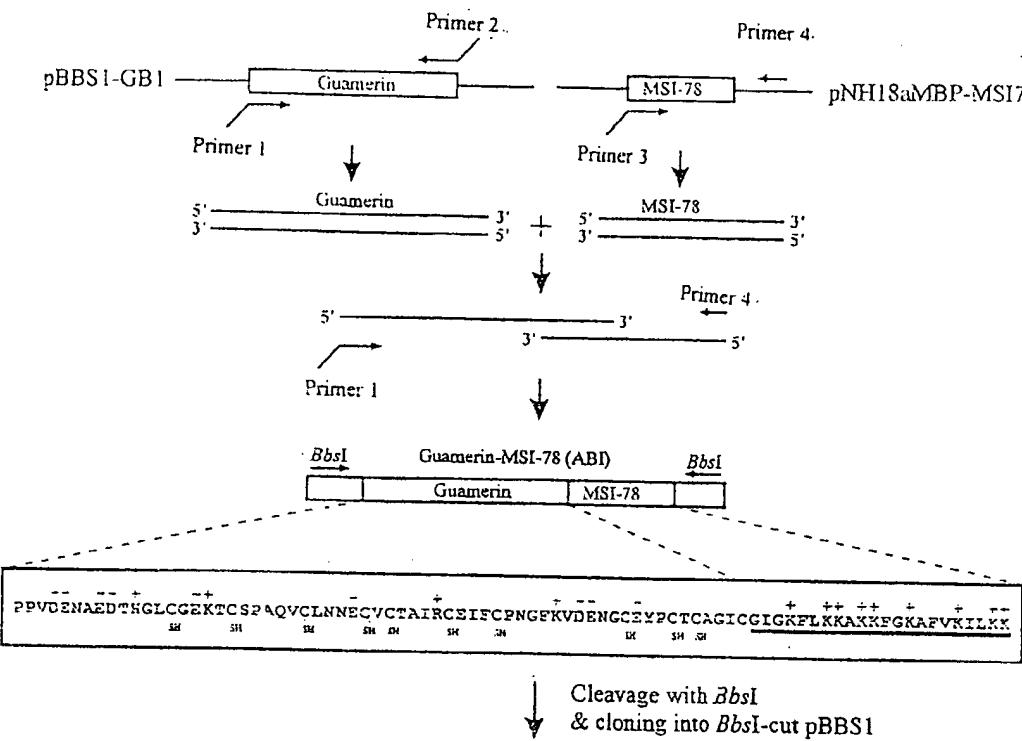


FIG. 4 (A)

pBBS1-(ABI)

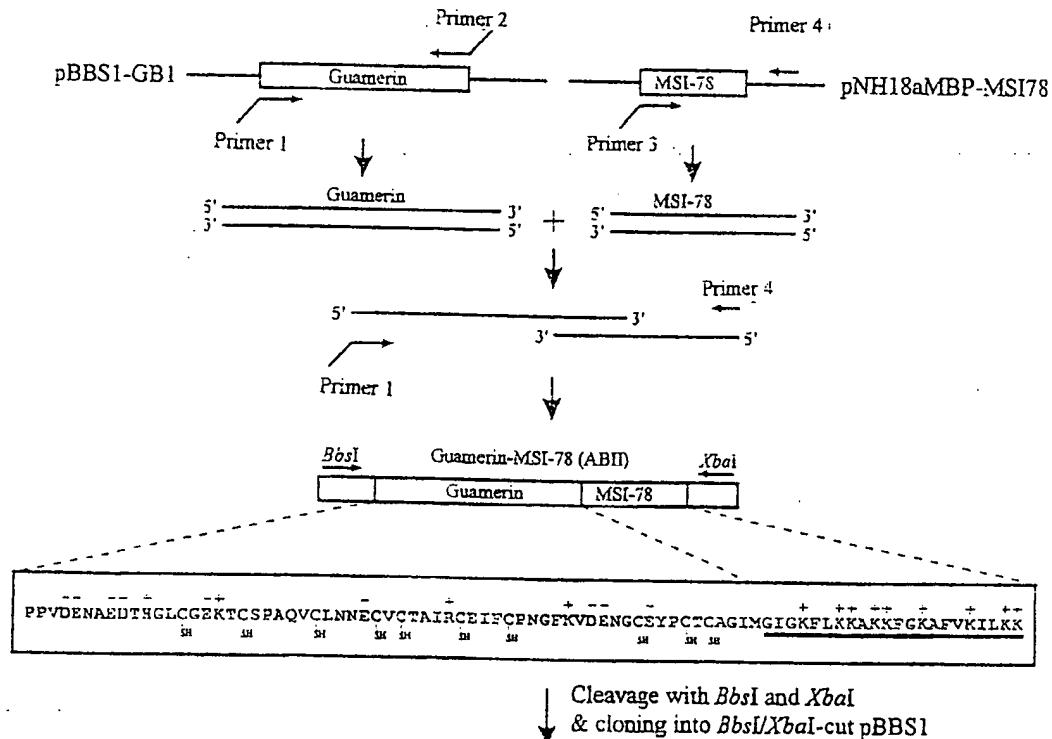


FIG. 4 (B)

pBBS1-(ABII)

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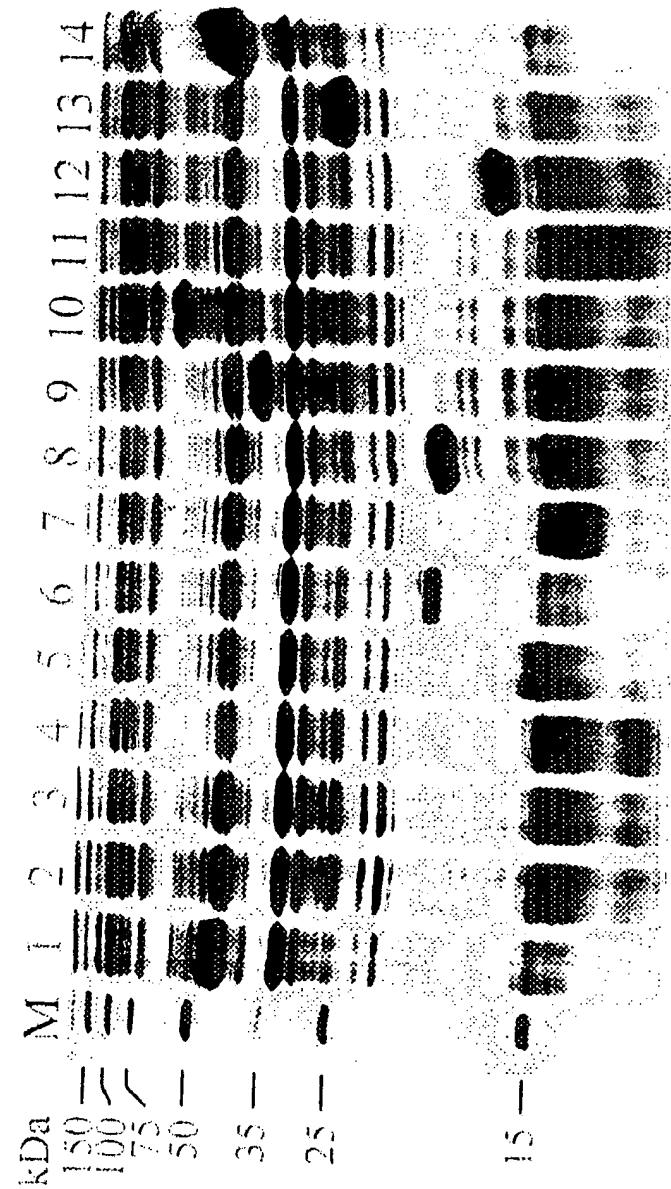


FIG. 5 (A)

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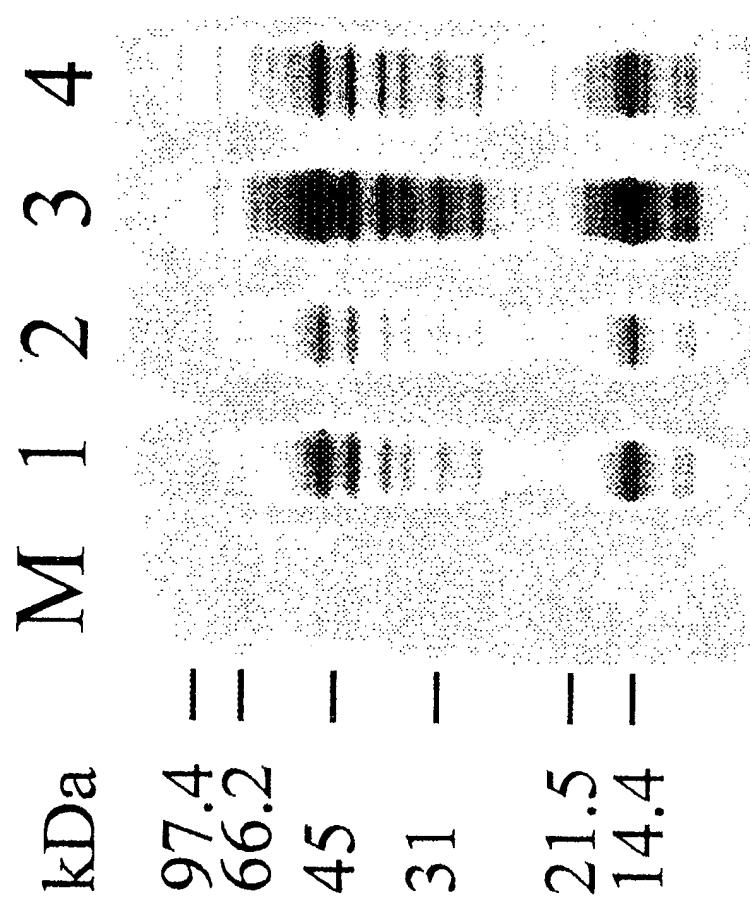


FIG. 5 (B)

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M 1 2 3

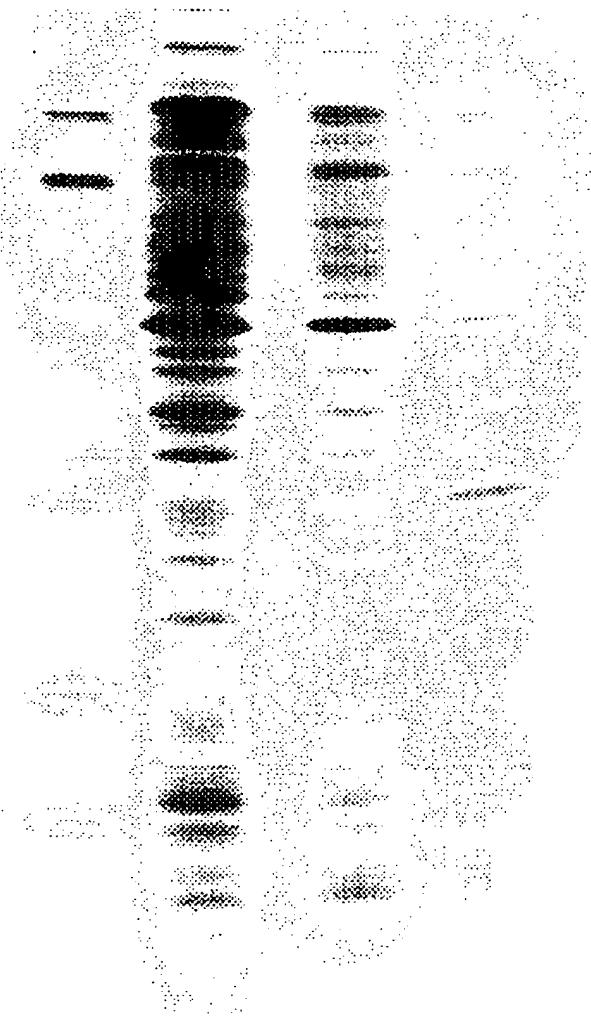


FIG. 6

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M 1 2 3 4 5 6 7 8

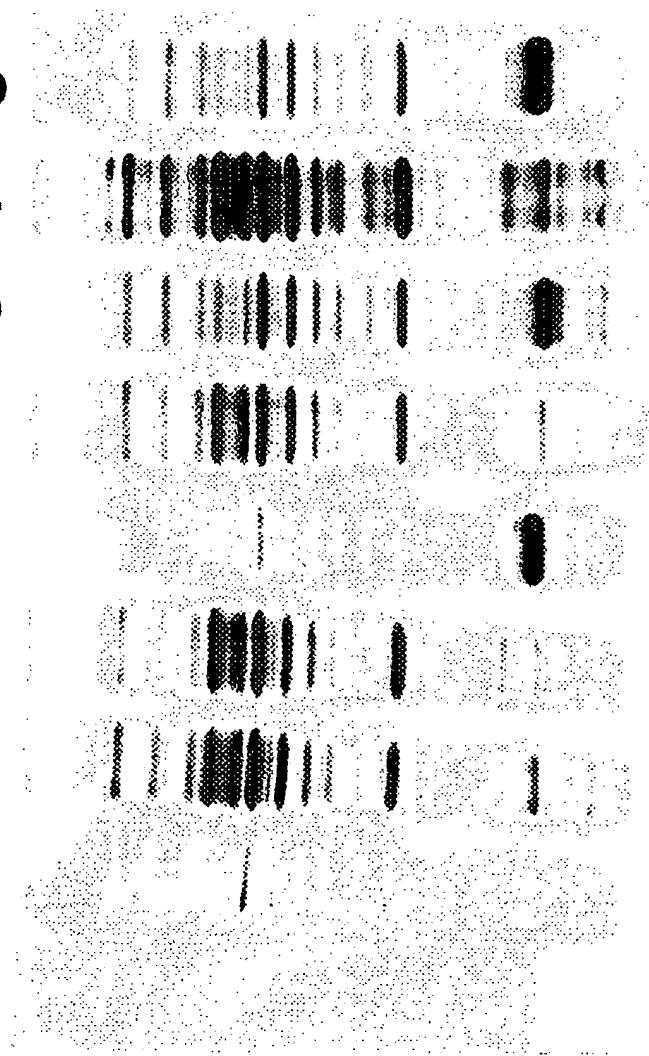


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 98/00132

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁶: C 12 N 15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁶: C 12 N 15/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| A | US 5 593 866 A (HANCOCK et al.) 14 January 1997 (14.01.97), claim 1. ----- | 1 |

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search

21 August 1998 (21.08.98)

Date of mailing of the international search report

08 September 1998 (08.09.98)

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 98/00132

| Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche | Datum der Veröffentlichung Publication date Date de publication | Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets | Datum der Veröffentlichung Publication date Date de publication |
|--|--|---|--|
| US A 5593866 | 14-01-97 | EP A1 656952 JP T2 8501213 WO A2 9404688 WO A3 9404688 US A 5688767 US A 5707855 US A 5789377 CA AA 2215362 EP A1 815247 WO A1 9628559 | 14-06-95 13-02-96 03-03-94 15-09-94 18-11-97 13-01-98 04-08-98 19-09-96 07-01-98 19-09-96 |